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Analysis of PEG 400 and 4000 in urine for gut permeability assessment using solid phase extraction and gel permeation chromatography with refractometric detection

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Abstract

We developed a treatment of urine samples allowing the analysis of two intestinal permeability markers: polyethylene glycol (PEG) 400 (highly diffusible; basal permeability indicator) and PEG 4000 (poorly diffusible; indicator of an abnormal increase of permeability) by a unique gel permeation chromatography (GPC) with refractometric detection. Urinary PEG were extracted using a mixed-bed resin composed of C2 and C18 layers. Permeability mean values determined in 11 human healthy subjects were 24.20 ± 9.30% and 0.12 ± 0.08% for, respectively, PEG 400 and 4000. The percentage of the PEG 4000 permeability value to the one of PEG 400 corresponded to an intestinal permeability index (IPI) of 0.52 ± 0.35 expressing a low diffusion of this poorly permeability marker.
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1. Introduction

Potential usage of in vivo measurements of human intestinal permeability covers a range of diagnostic applications such as (1) the follow-up of the onset of intestinal infection, as well as the prediction of the relapse of disease states of inflammatory bowel disease [1–9]; (2) the survey of food intolerance, allergy and eczema [10,11] and (3) the investigation of malignancy and chemotherapy side effects (for review, see [1]). Intestinal permeability is also affected in AIDS and cirrhotic patients [12], as well as during severe pancreatitis [13] and chronic renal failures [14]. Intestinal permeability could also be assessed in subjects regularly submitted to a treatment by non-steroidal anti-inflammatory drugs (NSAIDS), a condition known to lead to an abnormal increased permeability [15,16]. The determination of intestinal permeability generally consists in measuring urinary excretion rate of orally administered permeability markers, such as lactulose, polyethylene glycol (PEG), ^{51}Cr-EDTA. Polyethylene glycol (general formula $H(OCH_2CH_2)_nOH$) are linear polymers comprised of repeating ethylene oxide sub-units. As they are inert, stable to bacterial enzymes, non-toxic and easily excreted, these polymers are widely used as biocompatible materials in various formulations (such as food additives, sugar paste and purgative in preparation for colonoscopy). The validity of PEG as permeability markers for the paracellular route, and the contribution of this permeation pathway to quantitative intestinal absorption of small hydrophilic molecules have been extensively discussed in the literature (for review, see [17–19]). PEG 400 is the most frequently used PEG in human studies [1,2,10,12,14,15,20–31]. As they cover a wide range of molecular masses, high size PEG classes can be expected to simulate the transintestinal diffusion of macromolecules, such as allergens and endotoxin. Among those polymers, PEG exhibiting a molecular mass exceeding 3350 are considered to be poorly absorbable markers in intestinal perfusion studies [32]. This observation led some authors to select the PEG 4000 to assess the unusual rise of intestinal permeability to macromolecules associated with eczema, food allergy, rheumatoid arthritis, Crohn's disease and alcohol-induced liver disease [2,11,12,22].

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The simultaneous use of two test markers allows to express the global intestinal permeability as an index (IPI) reflecting the transfer value of the less permeable test marker (for example, PEG with Mr ≥4000) relative to the most diffusible probe (for example, PEG 400). Since pre-absorption factors (such as gastric emptying, dilution by digestive secretions) and post-absorption factors (such as systemic distribution and renal clearance) are assumed to affect both molecules equally, the value of this index should then be directly comparable from one individual to another, as already demonstrated with another category of permeability markers consisting of non-metabolisable sugars (for review, see [17]). To simultaneously measure intestinal permeability to small (PEG 400) and large (PEG 4000–10,000) PEG, Parlesak et al. [2,12] developed a liquid–liquid extraction procedure. This method enabled them to measured PEG 400 recovered in the aqueous phase by RP-HPLC, a method used by many authors to assessed PEG 400 concentrations in biological samples [12,15,22,27–31,33,34]. As large-sized PEG are not easily quantifiable by this chromatographic technique, PEG 4000 (and higher Mr PEG) extracted by the chloroform was determined using a permeation gel chromatography (GPC). Though accurate, a method requiring two different chromatographic analyses per sample is time consuming.

The present work has then been undertaken with the aim to develop a simple extraction procedure of urinary PEG, allowing the determination of PEG 400 and PEG 4000 under a unique chromatographic method (gel permeation chromatography). Several matrixes have been tested for their capacity to isolate each PEG class from urine contaminants that would impede their detection under chromatography.

2. Experimental

2.1. Materials

Pharmaceutical grade PEG 400 and 4000 used in permeability tests corresponded to Macrogolum 400 and 4000 (Alpha Pharma, distributed by CERTA, Belgium). PEG standards (Polymer Laboratories Inc, Amherst, MA, USA) used to set elution conditions, were: PEG 400 (PL 2070–3001; Mw/Mn = 1.05), PEG 1080 (PL 2057–5001; Mw/Mn = 1.04), PEG 4120 (PL 2070–7001; Mw/Mn = 1.02), PEG 8500 (PL 2070–9001; Mw/Mn = 1.03). Gel permeation columns (Hydrogels in series, namely: ^{TM}120, to, and ^{TM}250, to separate PEG with MW comprised between 1×10^3 Da and 8×10^4 Da) and corresponding guard column were purchased from Waters (Milleford, MA, USA), while RP-HPLC column used in this study was a LichroCART® 250-4 (Merck, Darmstadt, Germany). Solid phase matrixes and/or column used to extract PEG from urine were a silica gel (Silica gel 40, particle size 0.063–0.2 mm; 70–230 mesh ASTM, Merck), a cyanopropyl silica-based sorbent (CN-E; Varian, Palo Alto, CA, USA) or a mixed-bed resin C2/C18 (Isolute; International Sorbent Technology, Mid Glamorgan, UK). HPLC grade methanol and acetonitrile were used throughout the study (Labscan, Dublin, Ireland).

2.2. Subjects and urine samples

Urine samples used in this study were collected during 8 h from healthy volunteers fasted overnight (last meal before 8 p.m. the day before). Healthy subjects ingested a 100 ml permeability test solution containing 2 g of PEG 400 and 5 g of PEG 4000 in water. Each subject ingested the PEG solution before a standardized breakfast meal and was allowed to drink at will during all the experimentation period (8 h). Oral doses of PEG were set according to Parlesak et al. protocol [2,12]. The urine collection time was chosen after considering protocols covering 6 h [14,21,22,24,25,29,34] up to 24 h [2,10,14,23,31,36]. Subject comprised eleven adults (five men and six women, 23–40 years old, 52–65 kg), with no history of renal or gastrointestinal disorders. No subjects were used who consumed drugs (especially NSAID) or alcohol. The lunch meal was also standardized. Urine samples gathered over 8 h in clean plastic bottles devoid of preservative agents have been kept frozen (−20 °C) until processing for analysis (within 6 months). PEG ingestion was made under approval of the local ethics committee (Faculty of Medicine of the University of Liege, file no. 2001/4). All involved persons gave their informed consent to participate in the study.

The intestinal permeability value is expressed as the percentage of the marker (PEG 400 or 4000) oral dose excreted during 8 h. The intestinal permeability index is an expression of the PEG 4000 intestinal permeability, relative to PEG 400 one (%).

2.3. Apparatus

Chromatographic system consisted of a Waters 2690 separation module equipped with 1 gel permeation column (Waters 120^{TM}, to elute separately PEG 400 and 4000) or 2 gel permeation columns in series (Waters ^{TM}120 and ^{TM}250, when PEG Mr extended up to 10,000), following a guard column. The mobile phase was ultra-pure water driven through the system at a flow rate of 0.8 ml/min (isocratic condition) and eluted PEG were detected using a refractometer heated at 40 °C. As mentioned above, certain fractions containing PEG 400 (non-extracted urine samples or extraction fractions bearing high amounts of urinary contaminants) were analysed by RP-HPLC, using 20% ethanol as mobile phase (at a flow rate of 0.8 ml/min) at room temperature. All columns were alternatively injected with high and low amounts of standard PEG to check for possible column carryover in the concentration ranges used for either standard curves (see below) or spiking of analysed samples.

2.4. Experimental extraction method

The extraction procedure was preceded by a slow thawing of frozen samples favouring the formation of macroscopic

aggregates that were discarded by filtration (Whatman paper filter no.12). For standard curve establishment, PEG (from 5 mg/ml stock solutions, realised in milliQ water at 40 °C for 20 min and kept at room temperature for 1 month) were added to urine samples to obtain a final concentration ranging from 1 to 60 μg/ml for PEG 4000 and 12–3500 μg/ml for PEG 400. All samples were then submitted to the extraction method, consisting of a solid phase extraction (SPE) performed by a (1) cyanopropyl silica-based sorbent (CN-E); (2) silica gel (Silica gel 40, particle size 0.063–0.2 mm; 70–230 mesh ASTM, Fluka) put in a 5 ml syringe and pre-incubated with acetate buffer (0.1 M, pH 5.3) or (3) a mixed-bed resin (C2/C18) consisting of one layer of ethyl silica-based sorbent (C2) covering layer of octadecyl silica-based (end-capped) sorbent [C18(EC)].

During the extraction procedure development, the same experimental set was applied with all these solid phases, namely: the sorbent hydration (with milliQ water: 3 × 1 ml for PEG 400 series; 5% methanol for PEG 4000 series), followed by (1) the sample load of 1 ml for PEG 400 extraction or 10 ml for PEG 4000 extraction; (2) two sorbent washing steps with 1 ml (of milliQ water for PEG 400 series; of 5% methanol for PEG 4000 series); (3) a first PEG elution by 20% methanol (1 ml); (4) a second PEG elution by 40% methanol (1 ml); (5) a third PEG elution by 100% methanol (1 ml) and (6) a fourth PEG elution by 100% acetonitrile (1 ml).

The filtration of all these solvents and/or samples through the solid phase was driven by a mild centrifugation (1000 × *g* for 2 min, at room temperature) and the resulting filtrates were numbered from 1 (issued from the load of the sample) to 6 (issued from the elution by 100% acetonitrile). Filtrates 1 and 2 were freeze-dried. Methanol contained in fractions 3 and 4 was evaporated (centrifugation in a Speed Vac, Savant SC 100) before freeze-drying, while organic solvents (a mixture of methanol and acetonitrile) from fractions 5 to 6 were directly dried by centrifugation performed under vacuum (Speed Vaac). Dried residue from fraction 5 and 6 were dissolved in 200 μl of water, while residues from fractions 1 to 4 were dissolved in 1 ml water (only fractions 3 and 4 were clean enough to be analysed by GPC. When needed, fractions 1 and 2—containing high amounts of urine contaminant co-eluting with PEG under GPC—were analysed by RT-HPLC). All water-reconstituted samples were filtered through a nylon acrodisc syringe filter (diameter: 13 mm, porosity 0.2 μm; Gelman) before injection (75 μl) on the chromatographic system (GPC).

2.5. Optimised extraction method

The PEG extraction with the C2/C18 Matrix as described above (Section 2.4), was further improved using a procedure consisting in the succession of the following steps: (1) hydration of the mixed-bed resin with 40% methanol (1 ml); (2) sample load (1 ml to determine PEG 400, 10 ml to determine PEG 4000), (3) PEG 400 elution with 60% methanol (1 ml) and (4) PEG 4000 elution with successively 1 ml of 100% methanol and (5) PEG 4000 final elution with 1 ml acetonitrile.

The filtration of all these solvents and/or samples through the solid phase was driven by a mild centrifugation (1000 × *g* for 2 min, at room temperature) and the resulting filtrates were numbered from 1 (issued from the load of the sample) to 4 (issued from the elution by 100% acetonitrile). Methanol contained in the PEG 400 enriched-fraction (fractions 2) was evaporated (centrifugation in a Speed Vac, Savant SC 100) before freeze-drying, while organic solvents from PEG 4000 enriched-fractions (pooled fractions 3 and 4) were directly dried by centrifugation performed under vacuum (Speed Vaac). Dried residue from PEG 4000 fraction was dissolved in 200 μl of water, while residues from fraction 2 (PEG-400 enriched) were dissolved in 1 ml water. Water reconstituted samples were filtered through a nylon acrodisc syringe filter (diameter: 13 mm, porosity 0.2 μm; Gelman) before injection (75 μl) in the GPC column. PEG concentration of urine sample from permeability tests was determined comparing the detected peak to the one obtained after spiking samples with known amount of each PEG (i.e. 300 μg/ml of PEG 400 and 25 μg/ml of PEG 4000) before extraction.

3. Results

3.1. Method development

The analysis of PEG contained in biological fluids (such as urine samples) by gel permeation chromatography, requires a prior cleaning of the material to be assayed. Indeed, aqueous fractions from the liquid–liquid extraction of urine samples contained high amounts of urinary contaminants that co-eluted especially with PEG 400 under GPC (data not shown). For this reason, non-decontaminated samples had to be analysed by reverse phase chromatography RP-HPLC. As it would be more helpful to determine low- and high-sized PEG markers by a unique gel permeation chromatography, we tested several solid phases (or SPE columns) for their ability to clean urine fractions containing PEG 400. To this end, we first investigated the competence of a matrix showing an intermediate property between the high polar reverse phase (C18, for example) and a polar one (non-grafted silica resin) to extract PEG, from either urine samples loaded with known concentrations of PEG 400 and 4000. However, the corresponding resin, a cyanopropyl sorbent, did not retain the PEG 400 which was recovered in the first filtrates gathered after the sample loading and the subsequent washing of the SPE column (Fig. 1, fractions 1 and 2). As these fractions are enriched with contaminant of the urine that co-elute with PEG 400 under GPC, they had to be analysed by RP-HPLC. By contrasts, the material (PEG 4000) eluted by 40–100% methanol (Fig. 1, fractions 4 and 5) was contaminant-free and then determined by GPC.

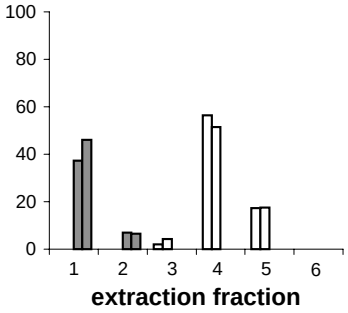

Fig. 1. Distribution of PEG 400 (grey columns) and PEG 4000 (white columns) (as % recovered after extraction) among extraction fractions obtained by filtration performed in duplicate through a cyanopropyl sorbent (CN-E) of a 5 ml urine sample containing 0.5 mg of both PEG 400 and 4000. Fractions 1–6 corresponded, respectively to: (1) the sample load; (2) the matrix washing; (3) the elution with 20% methanol; (4) the elution with 40% methanol; (5) the elution with 100% methanol and (6) the elution with 100% acetonitrile. Urine fractions 1 and 2 contained contaminant levels impeding the PEG 400 determination by GPC (theses fractions were analysed by RP-HPLC).

We also investigated a polar solid phase, a silica gel, pre-conditioned at acidic pH, using the same extraction procedure. Interestingly, the PEG 400 was selectively adsorbed and then desorbed (by adding successively 20% methanol, 40% methanol, 100% methanol and 100% acetonitrile, Fig. 2A, fractions 3–6) from the silica matrix in fractions devoid of urine contaminants and then analysed under GPC (Fig. 2B). However, the more hydrophobic PEG 4000 was recovered in none of the analysed fractions and this method appeared then to be inappropriate for the simultaneous extraction of both PEG classes.

3.2. Method optimisation

On the basis of the results described above, it appeared that the extraction of both PEG classes from a same solution requires the successive use of two solid phases, one showing adsorption selectivity for PEG 4000 and the other successfully adsorbing PEG 400 without retaining urinary contaminants. Interestingly, the combination of a highly non-polar matrix (for the retention of PEG 4000) and a weakly non-polar one (for the retention PEG 400) was available as one SPE column where matrixes C18 and C2 are overlaid. When performing the extraction procedure, this mixed-bed resin retained 85% of the loaded PEG 400 (Fig. 3A, fractions 2–4) that selectively eluted from the matrix by 20 and 40% methanol in contaminant-free fractions (Fig. 3A, fractions 3 and 4). The remaining part of urinary PEG 400 (30% that can not be analysed by GPC) was lost in the two first fractions successively obtained after the loading of PEG samples and the washing of the solid phase (Fig. 3A, fractions 1 and 2) and had then to be analysed under RP-HPLC. It has to be noted that, compared to results obtained for PEG 400 determination by GPC, the RP-HPLC method seemed to underestimate PEG 400 in fraction 3 and 4 of urine samples (compare fractions 3 and 4 for both

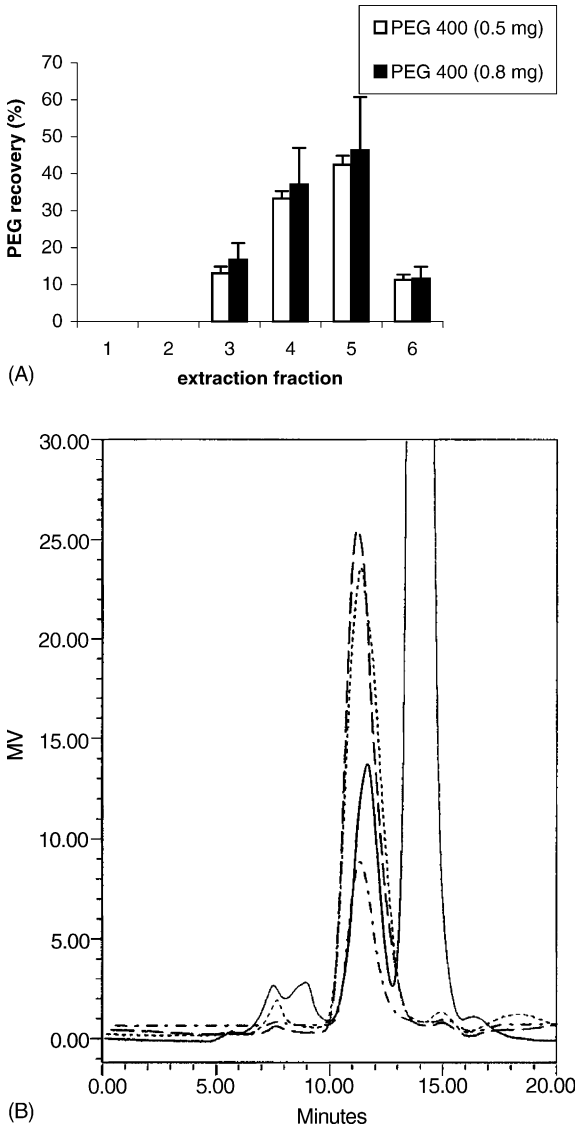

Fig. 2. (A) PEG 400 distribution (as % recovered after extraction) among extraction fractions 1–6 obtained from the filtration through a silica-gel sorbent of two urine samples (5 ml), bearing, respectively, 0.5 and 0.8 mg of both PEG 400 and PEG 4000. Results are expressed as mean value ± standard deviation, for five different extractions. (B) GPC chromatograms of PEG contained in fractions 3 (solid line), 4 (dotted line), 5 (dashed line) and 6 (dashed dotted line) obtained from the filtration of one urine sample through a silica gel sorbent. The peak eluted at a retention time (RT) of 10.87 min, correspond to the RT of PEG 400 standard eluted under the same conditions (see standard PEG 400, in (A)). The chromatography was performed using a gel permeation column Waters ™120 and milliQ water as mobile phase at a flow rate of 0.8 ml/min.

methods in Fig. 3A). The extraction process was efficient for both PEG between 0 and 60–400 μg/ml (see overlaid chromatograms of spiked samples in Fig. 4A and B). However, the correlation coefficient obtained for the standard curve of PEG 4000 in urine was not satisfactory (0.975, equation not shown) and the extraction procedure was then improved to get a better recovery of this polymer from urine samples. The amendments brought to the extraction method affected

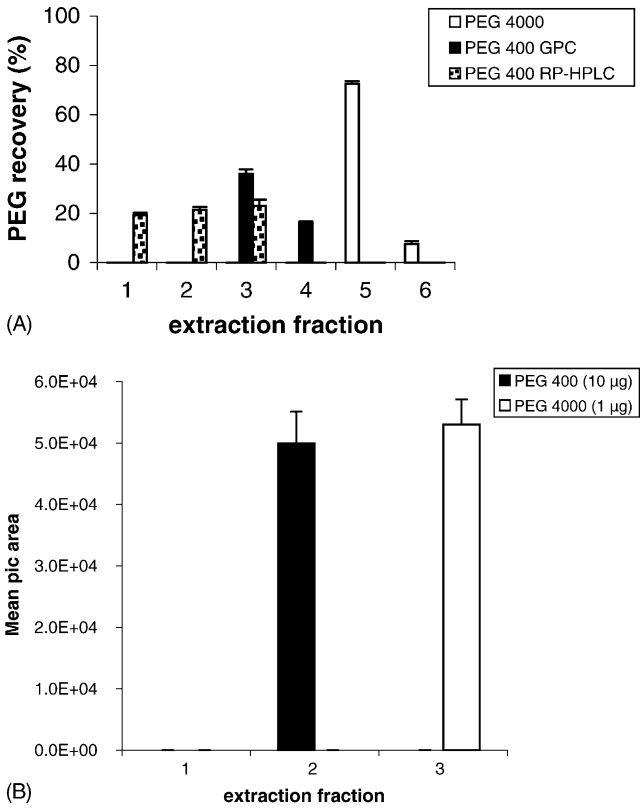

Fig. 3. (A) Distribution profiles of PEG 400 and PEG 4000 (as % recovered after extraction) among extraction fractions obtained from filtration through a mixed-bed resin C2/C18 of a 5 ml of standard solution of both PEG in urine (0.5 mg/ml of each PEG). Results are expressed as mean ± standard deviation, for four different extractions. Fractions 1–6 corresponded to, respectively: (1) the sample load; (2) the matrix washing; (3) the elution with 20% methanol; (4) the elution with 40% methanol; (5) the elution with 100% methanol and (6) the elution with 100% acetonitrile. PEG 4000 was determined by GPC in fractions 5 and 6 of each sample series. PEG 400 was only measured by GPC in fractions 3 and 4. (B) Distributions of PEG 400 (fraction 2) and PEG 4000 (fraction 3) obtained from filtration through a mixed-bed resin C2/C18 of urine containing 240 μg (1 ml) of PEG 400 or 30 μg (10 ml) of PEG 4000. Results are expressed as mean peak area ± standard deviation for five different extractions. Fractions 1–3 corresponded, respectively to: (1) the sample load (1 ml to determine PEG 400; 10 ml to determine PEG 4000), (2) PEG 400 elution with 60% methanol (1 ml) and (3) PEG 4000 elution with successively 1 ml of 100% methanol and 1 ml of acetonitrile. NB: PEG 400 was not quantifiable by GPC in fraction 1 (enriched in urine contaminants).

(1) the conditioning of the solid phase (in 40% methanol instead of water); (2) the washing step of the matrix following the sample loading (omitted) and (3) the PEG 400 elution conditions (see 2.5). As an advantage this optimised method only produced three fractions, with fraction 2 and 3, respectively, enriched in PEG 400 and PEG 4000 (Fig. 3B).

3.3. Resolution, linearity and sensitivity of the optimised method

The precision of the method was assessed by repeated extractions of PEG 4000 (10 μg/ml) and PEG 400 (250 μg/ml)

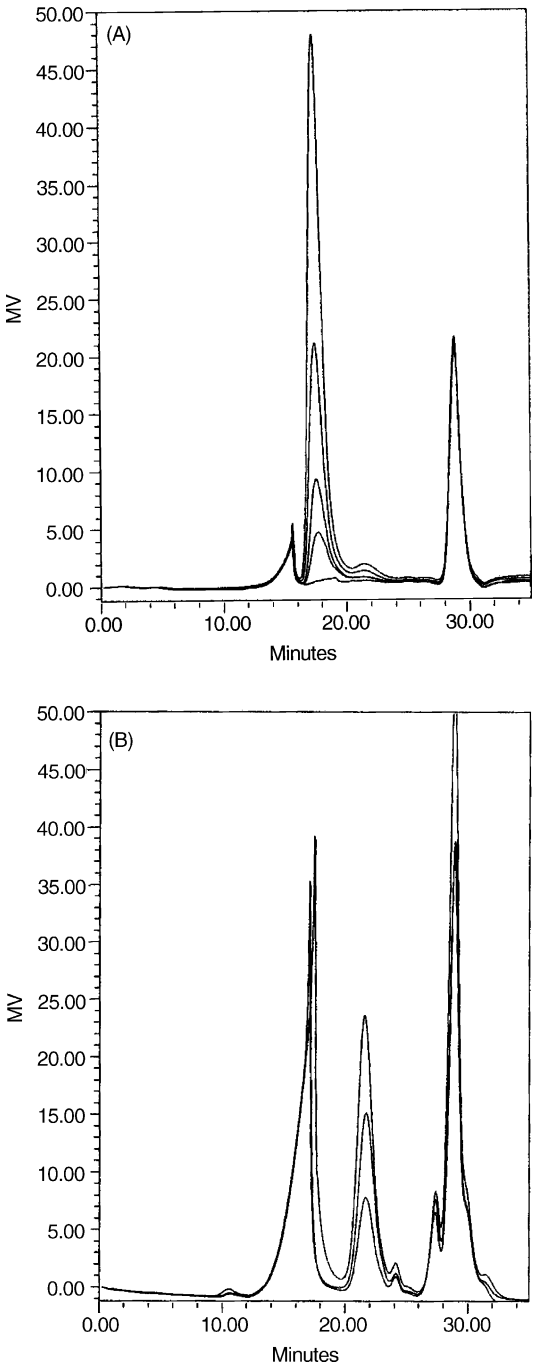

Fig. 4. (A) GPC chromatograms of PEG 4000 (RT 18.13 min) contained in the pooled fractions 5 and 6 obtained from the filtration of five urine samples through a C2/C18 sorbent. Starting from the baseline towards the tip of the highest peak, there is a urine sample devoid of PEG 4000, and then the same urine sample added of increasing amount of PEG 4000 (successively 5, 10, 20 and 40 μg/ml) before extraction. The chromatography was performed using two gel permeation columns Waters ™120 and Waters ™250, with milliQ water as mobile phase at a flow rate of 0.8 ml/min. (B) GPC chromatograms of PEG 400 (RT 22.16 min) contained in the pooled fractions 3 and 4 obtained from the filtration of four urine samples through a C2/C18 sorbent. Starting from the baseline towards the tip of the highest peak, there is one urine sample added of respectively 25, 50 and 75 μg/ml of PEG 400. The chromatography was performed using two gel permeation columns Waters ™120 and Waters ™250, with milliQ water as mobile phase at a flow rate of 0.8 ml/min.

Table 1
Linear regression equations and correlation coefficients of calibration curves, determined for PEG 400 and 4000 extracted in triplicates by the C2/C18

PEG Mr	Concentration range (μg/ml)	Linear equation	Correlation coefficient
400 in water	0–400	$Y = 1560x + 6135$	0.999
400 in urine	0–400	$Y = 1361x + 14034$	0.996
4000 in water	0–75	$Y = 94017x - 40102$	0.997
4000 in urine	0–75	$Y = 57424x - 66992$	0.987

Tested concentration were 0, 5, 15 30, 40, 50, 60 and 70 μg/ml for PEG 400 and 0, 25, 100, 200, 250, 300, 350 and 400 μg/ml for PEG 4000.

added one urine sample (within series precisions). The coefficient of variation for PEG determination in urine was 3.8% ($n = 5$) for PEG 4000 and 5.3% ($n = 8$) for PEG 400. Compared to the extraction of PEG dissolved in water, recovery of PEG dissolved in urine was comprised between 72.93 and 87.21% for PEG 400 and between 62.86 and 95.07% for PEG 4000, depending on the urine sample. The urine composition (whose osmolatility, and consequently salt composition, could vary from 500 to 1400 mmol/kg water) was shown to equally affect the retention properties of both polymers.

Standard curves (Table 1) of PEG 400 and PEG 4000 were prepared separately in the range of 25–400 μg/ml for PEG 400 (tested concentrations: 0, 25, 100, 200, 250, 300, 350 and 400 μg/ml) and 5.0–70 μg/ml for PEG 4000 (tested concentrations: 0, 5, 15 30, 40, 50, 60 and 70 μg/ml). These concentrations ranges were chosen in accordance with the expected values of each polymer in urine samples. We also checked that the linear relationship between the urinary load of PEG 400 and the peak intensity under GPC extended as far as 3.5 mg/ml, a concentration that could be reached in urine samples collected during 8 h following the ingestion of 2 g of this substance (Table 2). The correlation coefficient of each curve was superior or equal to 0.99 (Table 1), indicating a linear relationship between polymer concentration and detector response. Given that assay sensitivity can be defined as the smallest detectable concentration yielding a signal-to-noise ratio 3:1, the minimal amount limit of PEG 400 was 25 μg/ml, while the one of PEG 4000 was 5 μg/ml. These detection limits appeared to be under the mean urinary concentration (1.15 mg/ml of PEG 400 and 9.71 μg/ml of PEG 4000, 8 h after the ingestion of an oral dose, see Table 2).

3.4. Measurement of intestinal permeability of healthy subjects

As a first application of the whole extraction-determination method, we performed an intestinal permeability test, using oral doses of PEG 400 and 4000 identical to those applied by Parlezak and co-workers [2,12] (i.e. 2 g of PEG 400, 5 g of PEG 4000) in healthy subjects. Mean values for PEG 400 and PEG 4000 permeabilities (expressed as a percentage of PEG recovered in the whole urine samples gathered during 8 h following the ingestion) were, respectively, 24.2 ± 9.3 and 0.115 ± 0.08 (Table 2). The calculated permeability index (IPI, in %) in healthy subjects, corresponding to the expression of PEG 4000 permeability relative to that of PEG 400 amounted to 0.519 ± 0.349, reflected the expected low diffusion of PEG 4000.

4. Discussion

Intestinal permeability tests involving PEG as permeability probes, instead of small sugars offer two major advantages. Firstly, these polymers cover a wide range of molecular sizes, including those of macromolecules (such

Table 2
PEG in urine samples gathered over 8 h from healthy subject (HS 1–HS 11) following the ingestion of 2 g of PEG 400 and 5 g of PEG 4000

Healthy subject	PEG 400			PEG 4000			IPI
	Tot excr (mg)	Ur conc (mg/ml)	Perm (%)	Tot excr (mg)	Ur conc (μg/ml)	Perm (%)	
HS 1	451.05	0.87	22.55	1.95	3.76	0.039	0.173
HS 2	270.47	0.53	13.52	5.20	10.09	0.104	0.769
HS 3	332.43	0.46	16.62	3.34	4.62	0.067	0.401
HS 4	397.65	1.02	19.88	8.80	22.67	0.176	0.885
HS 5	495.17	0.50	24.76	15.49	15.65	0.310	1.251
HS 6	245.08	0.49	12.25	3.44	6.93	0.069	0.561
HS 7	850.11	2.24	42.51	6.65	17.51	0.133	0.313
HS 8	675.10	2.60	33.76	2.55	7.09	0.051	0.151
HS 9	386.60	1.93	19.33	4.28	9.31	0.086	0.443
HS 10	616.47	1.30	30.82	1.91	4.03	0.038	0.124
HS 11	603.67	0.72	30.18	9.76	11.68	0.195	0.646
Mean	483.98	1.15	24.20	5.76	10.30	0.115	0.519
Standard deviation	186.07	0.77	9.30	4.17	6.11	0.083	0.349

For each PEG, results are expressed as total excretion (tot excr; μg), urinary concentration (ur conc; mg/ml for PEG 400 and μg/ml for PEG 4000) and permeability (perm, %). The intestinal permeability index (IPI) was established.

as allergens). Secondly, their chromatographic determination is unaffected by the presence of sugars found in the food and urine and then does not requires an adaptation of subject diet prior to the test application. However, though they fit all requirements of permeability markers, the systematic use of PEG mixture to assess intestinal permeability is hampered by the technical complexity inherent in the need to use different analytical methods when determining either low or high size molecules. In this study, we attempted to overcome this shortcoming by developing an extraction procedure allowing the analysis of urinary PEG 400 and PEG 4000 by a unique chromatographic technique (GPC).

The first sorbent selected for a SPE extraction of PEG contained in urine sample, a cyanopropyl solid phase, was not efficient to isolate PEG 400 from the urinary contaminants that impede their detection under GPC. The second sorbent tested was silica gel, that proved to be efficient to only extract PEG 400 from urine samples. Consequently both sorbents should be used in series for the determination of PEG 400 and 4000.

Alternatively, a successful extraction of PEG 400 and PEG 4000 from urine was achieved using a mixed-bed resin composed of overlaid C18 and C2 matrixes. This hydrid matrix allowed the production of extraction fractions presenting enrichment in PEG 400 or PEG 4000, while being depleted of interfering contaminants that would otherwise impede their determination by GPC. To our knowledge, results presented in the present paper constitute the first report of a simple extraction method permitting the analysis of urinary PEG 400 by gel permeation chromatography. Ryan et al. [35] also proposed an extraction method leading to the GPC determination of both PEG 3350 and PEG 400. However, their protocol required the use of two devices (size-exclusion membranes in combination with mixed ion exchange resin), instead of one in the present study. Using the combined SPE extraction/GPC determination described in the present paper, we obtained PEG 400 urinary concentrations falling into range values reported previously [2,12] under identical ingestion conditions. Our results are also in accordance with PEG 400 permeability values (IP) reported in other studies performed with different initial oral doses (for example, 1–15 g, IP = 21.9–27.1% after 6 h [4]; 10 g, IP = 30.1 ± 3.87% after 6 h [15]). Regarding the PEG 4000, permeability values obtained in the present study are higher than those published by Parlezak (0.115 ± 0.083 over 24 h versus 0.519 ± 0.349 over 8 h in the present study) but in agreement with values reported by Jackson et al. [36] (0.39±2.67), pointing out the suitability of the extraction method proposed in the present paper.

In relation with the PEG 4000 permeability level, the intestinal permeability index reported here is higher than the one we estimated from data published by Parlesak for healthy subjects (0.147 [2] or 0.108 [12]). However, by comparison to the IPI estimation we made from Parlesak data for Crohn's disease patients (1.34 [2]) and two groups of cirrhotic patients affected by alcohol abuse (3.551 and 4.176, respectively, [12]), IPI values determined in the present study (0.519) are in agreement with the one expected in healthy subjects.

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